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Simplified Radioimmunoassay for Detection of Human Rotavirus in Stools

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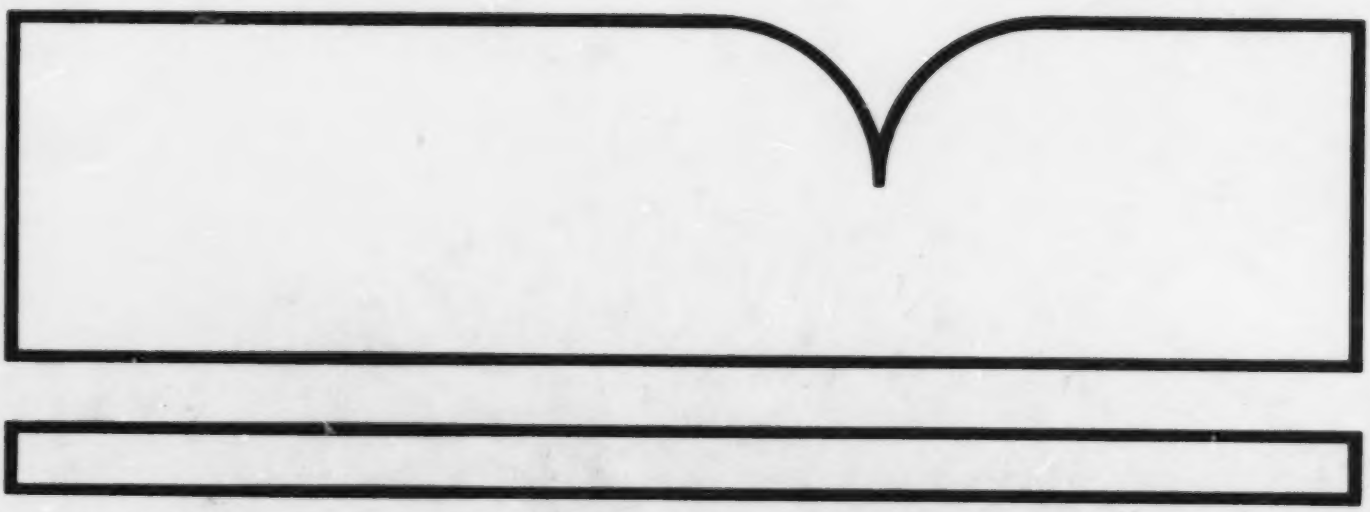

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16. ABSTRACT

A simplified radioimmunoassay (RIA) technique was developed to facilitate the diagnosis of human rotavirus in stools of infants with diarrhea. This microtiter solid-phase RIA utilizes as a critical reagent hyperimmune serum against a tissue culture-grown simian rotavirus that is immunologically closely related to the human agent. One hundred thirteen pediatric stool samples were tested. Thirty-five specimens known to be positive for rotavirus by electron microscopy were also positive by RIA. All RIA-positive reactions (36) were shown to be specific for rotavirus by means of an antibody-blocking test. Nonspecific RIA-positive reactions were not encountered. This accurate, sensitive RIA technique is practical for use in diagnostic laboratories because critical serum reagents are prepared against culture-grown virus and not against rotavirus derived from carefully selected stools of humans or experimentally infected gnotobiotic animals.

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Journal Article

Simplified Radioimmunoassay for Detection of Human Rotavirus in Stools

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A simplified radioimmunoassay (RIA) technique was developed to facilitate the diagnosis of human rotavirus in stools of infants with diarrhea. This microtiter solid-phase RIA utilizes as a critical reagent hyperimmune serum against a tissue culture-grown simian rotavirus that is immunologically closely related to the human agent. One hundred thirteen pediatric stool samples were tested. Thirty-five specimens known to be positive for rotavirus by electron microscopy were also positive by RIA. All RIA-positive reactions (36) were shown to be specific for rotavirus by means of an antibody-blocking test. Nonspecific RIA-positive reactions were not encountered. This accurate, sensitive RIA technique is practical for use in diagnostic laboratories because critical serum reagents are prepared against culture-grown virus and not against rotavirus derived from carefully selected stools of humans or experimentally infected gnotobiotic animals.

Human rotavirus is an important cause of pediatric diarrhea [1, 2]. Diagnosis of infection is difficult, because the virus fails to undergo complete replication in cell culture. Electron microscopy (EM) of diarrheal stool specimens is a reliable diagnostic technique for rotavirus but is cumbersome for routine diagnosis or large-scale studies. Two more suitable methods, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), have recently been reported to be as sensitive as EM in the detection of rotavirus in stool specimens [3–5]. In both of these techniques, hyperimmune animal serum that reacts with rotavirus is a critical reagent. Serum has been prepared either against rotavirus purified from human stools [4] or from the stools of gnotobiotic calves experimentally infected with human rotavirus [3, 5]. These reagent sera are difficult to prepare in most viral diagnostic laboratories because it is necessary to hyperimmunize animals either with a carefully selected preparation of human stool containing a high titer of rotavirus or with stool collected from experimentally infected gnotobiotic animals.

Another potential problem with existing assays is that the hyperimmune animal serum reagents have been described as possessing occasional nonspecific reactivity, presumably against extraneous stool components [3]. This report describes the development of an RIA technique for detection of human rotavirus in stool specimens with use of antiserum prepared against a tissue culture-grown simian rotavirus that is immunologically closely related to human rotavirus. The technique is specific, sensitive, and practical for the detection of human rotavirus in pediatric stool specimens.

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Materials and Methods

Stool specimens. Stool specimens tested by RIA were collected from 113 infants and young children in Taiwan and the Philippines. Specimens were obtained from 95 patients admitted

to the hospital with diarrhea and from 18 unhospitalized children without diarrhea of the same approximate age, sex, and socioeconomic background as the hospitalized patients. Details of the study populations, methods of collection, and EM characterization of specimens have been described [6, 7]. Stool suspensions (2%–5%) were prepared in veal infusion broth supplemented with 0.5% bovine serum albumin. Suspensions were clarified by centrifugation at 2,000 *g* for 15 min, filtered through a 1.2-μm membrane (Millipore Corp., Bedford, Mass.), and stored at −70 C until use.

Hyperimmune serum and immunoglobulin. Hyperimmune serum was prepared by inoculation of guinea pigs with high-titered, purified, tissue culture-grown SA11 virus, a simian rotavirus that is immunologically closely related to human rotavirus [8]. The procedure was as follows. SA11 virus [9] was provided by Dr. H. H. Malherbe (University of Texas Health Science Center, San Antonio, Tex.) and was grown in MA-104 cells, a continuous line of rhesus embryonic kidney cells (Flow Laboratories, Rockville, Md.). Cells were grown to confluency at 36 C in an equal mixture of Eagle's minimal essential medium and medium 199 supplemented with 10% heat-inactivated fetal calf serum and 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes) buffer. Cells were then washed twice with maintenance medium consisting of Earle's lactalbumin (0.5%) (Flow Laboratories) supplemented with 0.1% yeastolate (Difco, Detroit, Mich.), renewed with the same medium, and then infected with SA11 virus at a multiplicity of infection of 25 $TCID_{50}$ per cell.

Over 90% CPE was noted 48 hr later, and cultures were frozen and thawed three times and then clarified by centrifugation at 2,000 *g* for 15 min. The supernatant (1.5 liters containing 10^7 $TCID_{50}$/0.1 ml) was concentrated by ultrafiltration (Diaflo XM100A membrane, Amicon Corp., Lexington, Mass.) to a final volume of 30 ml containing 10^8 $TCID_{50}$/0.1 ml. Portions (5 ml) of this concentrated virus pool were applied to six 30-ml 20%–40% sucrose density gradients and centrifuged for 75 min at 82,000 *g* in a Beckman L2–65B ultracentrifuge with an SW27 rotor (Beckman Instruments, Fullerton, Calif.). Gradients were then fractionated into 1-ml portions. A distinct peak of ultraviolet light-absorbing material, appearing between fractions 10 and 15, was pooled and pelleted in a SW40 rotor at 200,000 *g* for 1 hr. The pellet was resuspended in 1.5 ml of phosphate-buffered saline (PBS) and was shown by EM to contain complete virus particles. This preparation had a viral titer of 10^9 $TCID_{50}$/0.1 ml, and a 260/280-nm absorption ratio of 1.3. The preparation was then mixed with an equal volume of incomplete Freund's adjuvant, and 0.4 ml was injected into the rear foot pads and thighs of each of five guinea pigs from which blood samples had been taken. Three weeks later, booster inoculations, prepared in the same manner, were administered in the front foot pads and thighs. Four weeks later animals were exsanguinated by cardiac puncture. Serum was separated, heat-inactivated at 56 C for 30 min, and titrated for levels of CF antibody to SA11 virus [10]. IgG was purified from serum by precipitation with $(NH_4)_2SO_4$ followed by ion-exchange chromatography [11].

Iodination. Purified guinea pig IgG was labeled with ^{125}I (about 10–100 atoms per molecule of IgG) with use of the chloramine T method of Hunter and Greenwood [12] with described modifications [3, 11]. $Na^{125}I$ (NEZ 033) was purchased from New England Nuclear, Boston, Mass. Labeled IgG was stored at 4 C and used within a week of its preparation.

RIA. A microtiter solid-phase RIA of the type described previously was used [3, 11]. Wells of microtiter plates (220-25, Cooke Engineering Co., Alexandria, Va.) were coated with 75 μl of hyperimmune guinea pig serum diluted 1:1,000 in PBS. This concentration was determined to be optimal by a preliminary titration of the coating antibody with SA11 virus. After absorption of coating antibody at 4 C for 4 hr, plates were washed twice with PBS, and the wells were filled with 1% bovine serum albumin in PBS. Plates were kept overnight at 4 C, washed twice with PBS, air-dried, sealed, and stored at −20 C until use.

Stool filtrate (75 μl) was incubated for 2 hr at room temperature (about 21 C) with 75 μl of a 1:5 dilution of normal guinea pig serum (obtained before immunization) that was previously shown not to inhibit SA11 virus reactivity in RIA; this serum also lacked CF antibody to SA11

virus. For blocking experiments a 1:5 dilution of hyperimmune guinea pig serum was used in place of the preimmunization serum. The serum-stool mixture (50 μl) was then placed in each of two precoated microtiter wells, and the plate was incubated for 48 hr at 4 C. Wells were then washed five times with PBS, and 50 μl of radiolabeled IgG, diluted in PBS to contain 2×10^5 cpm, was added to each well; the plate was reincubated for 4 hr at 37 C. (A dilution containing 2×10^5 cpm was determined to be optimal in a preliminary titration of ^{125}I-labeled antibody with SA11 virus.) Wells were again washed five times with PBS. The plates were then cut apart, and the amount of radioactivity bound to each well was determined in a gamma spectrometer (PRIAS, Packard Instruments, Downers Grove, Ill.).

To obtain RIA positive-to-negative (P/N) values, the counts of test samples were divided by the mean counts from six nondiarrheal stools known to be negative for rotavirus by EM. These stools were from unhospitalized control Taiwanese children described previously [6] and were not included in the 113 test specimens. The mean counts for these stools varied little in all five tests in which they were employed. Specimens were considered RIA-positive if they produced P/N ratios of >1.9 and also had P/N values reduced by >50% after preincubation with SA11 antiserum (blocking). RIA-positive samples showed counts of 435–4,354 cpm, whereas RIA-negative samples showed counts of 140–380 cpm.

Results

Selection of hyperimmune serum. Sera from the five hyperimmunized guinea pigs were evaluated in three ways: (*1*) the titer of CF antibody to SA11 virus; (*2*) P/N ratios obtained by reaction in RIA with cell culture-grown SA11 virus, where the N value represented uninfected culture medium; and (*3*) capability to distinguish by RIA between four stool specimens known to be rotavirus-positive by EM and four stool specimens known to be rotavirus-negative by EM. All sera possessed high titers of CF antibody, ranging from 1:4,096 to 1:16,384. All sera detected SA11 virus, with P/N ratios of 5.6, 10.1, 16.3, 21.5, and 37.0. The two sera with the highest P/N ratios readily identified all EM-positive and EM-negative stool specimens, while the three remaining sera showed some false-negative reactions. It therefore appears that the titer of CF antibody alone is an insufficient indicator of the suitability of a serum for RIA, an observation that has also been made in the selection of antiserum to human rotavirus for RIA (A. Kalica, personal communication). Based on the above evaluation, one guinea pig serum (CF titer of 1:8,192 and P/N ratio of 37.0 for detection of SA11 virus) was selected as the reagent for coating and iodination in the RIA test. The immune serum with a P/N ratio of 21.5 was used as the reagent in the blocking test. Serum taken before immunization from either of these two animals was used as the negative control for the blocking test.

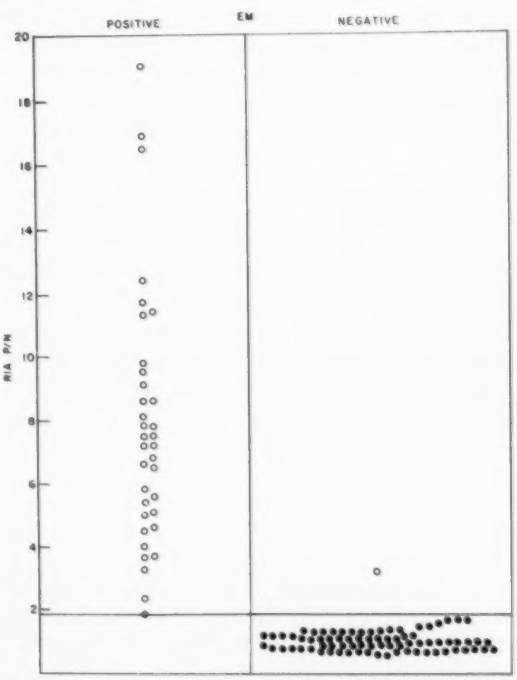

Figure 1. Distribution of positive-to-negative (P/N) values in radioimmunoassay (RIA) compared with results of electron microscopy (EM) for 113 pediatric stool samples. Samples exhibiting P/N values of >1.9 and also showing reduction of the P/N value by >50% after preincubation with immune serum (blocking) were considered RIA-positive. (○) = sample exhibited blocking; (●) = sample did not exhibit blocking.

Testing of human stools by RIA. Figure 1 shows results obtained by RIA examination of 113 pediatric stools. Thirty-six specimens were RIA-positive, demonstrating P/N values in the range of 1.9–19.1 (median, 7.2); all 36 samples were from children with diarrhea. Preincubation of all 36 specimens with SA11 antiserum (blocking) reduced P/N values in every instance by >50%. After blocking, P/N values ranged from 0.8 to 1.9 (median, 1.3). All RIA-positive samples except one were EM-positive for rotavirus. The one RIA-positive EM-negative specimen had its P/N value reduced by blocking by 53%, and this sample was regarded as falsely negative by EM.

Seventy-seven specimens were RIA-negative, showing P/N values in the range of 0.6–1.7 (median, 1.0) and these values were not reduced by preincubation with SA11 antiserum. All 77 samples were EM-negative for rotavirus; 18 of these samples were from children without gastroenteritis, and the remainder were from children with diarrhea. EM examination revealed adenovirus in four specimens and parvovirus-like particles in one specimen; all five of these samples were RIA-negative for rotavirus.

Repeated examination of all stool specimens yielded reproducible results; all samples retained their RIA positivity or negativity including appropriate blocking reactions.

Discussion

We have demonstrated that antiserum prepared against an antigenically closely related virus can be used efficiently in RIA to detect human rotavirus in stool specimens. In this regard it has been commented that high-titered guinea pig antisera prepared against two other related viruses (Nebraska calf diarrhea virus and the "O" agent) could not detect small amounts of rotavirus in human stools by ELISA [13]. Our studies indicate that detailed screening of several hyperimmune sera is required to select the critical test reagent. The data clearly show that a high titer of CF antibody is an insufficient single criterion for judging the efficiency of a serum in detecting rotavirus in stool samples.

This assay is highly specific. All EM-positive stool samples were also RIA-positive. Moreover, all RIA-positive reactions were shown to be specific for rotavirus by means of an antibody blocking test. We did not encounter nonspecific or extraneous antigen-antibody reactions (RIA false-positive results), which have been described with RIA systems that utilize serum reagents prepared against stool-derived rotavirus [3].

It has been noted that stool samples from Bangladesh must be preincubated with goat serum free of antibody to rotavirus to eliminate false-positive ELISA reactions with rotavirus antiserum prepared in goats [13]. These reactions have been attributed to the presence of antibodies to goat globulin in certain stools, presumably a result of widespread consumption of goat milk. In our RIA system that uses guinea pig serum, preincubation of the stools from our Far Eastern population with guinea pig serum free of antibody to rotavirus was required for elimination of false-positive results that otherwise would have occurred for three of 113 stool specimens. However, human serum that lacked fluorescence-stainable antibody to rotavirus [14] served equally well to eliminate the nonspecific binding of radioactivity by these three stool samples. Perhaps, then, an antibody to globulin crossing species lines is responsible for this nonspecific reaction. In practical terms, however, the possibility of this reaction is easily avoided in our RIA by preincubation of stools with guinea pig serum that lacks antibody to rotavirus.

This accurate, sensitive RIA test is practical for use in routine viral diagnostic laboratories, because critical serum reagents can be easily prepared against culture-grown virus rather than against high-titered rotavirus-positive stool preparations that must be carefully selected from stools of human infants or experimentally infected gnotobiotic animals. The assay also obviates nonspecific RIA-positive reactions that could complicate interpretation of results by diagnostic laboratories. It is likely that the critical serum reagents used in our RIA could also be adapted for use in an accurate, sensitive ELISA system.

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